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Divergent Gene Expression Following Duplication of Meiotic Genes in the Stick Insect *Clitarchus hookeri*

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Abstract

Some animal groups, such as stick insects (Phasmatodea), have repeatedly evolved alternative reproductive strategies, including parthenogenesis. Genomic studies have found modification of the genes underlying meiosis exists in some of these animals. Here we examine the evolution of copy number, evolutionary rate, and gene expression in candidate meiotic genes of the New Zealand geographic parthenogenetic stick insect *Clitarchus hookeri*. We characterized 101 genes from a de novo transcriptome assembly from female and male gonads that have homology with meiotic genes from other arthropods. For each gene we determined copy number, the pattern of gene duplication relative to other arthropod orthologs, and the potential for meiosis-specific expression. There are five genes duplicated in *C. hookeri*, including one also duplicated in the stick insect *Timema cristinae*, that are not or are uncommonly duplicated in other arthropods. These included two sister chromatid cohesion associated genes (*SA2* and *SCC2*), a recombination gene (*HOP1*), an RNA-silencing gene (*AGO2*) and a cell-cycle regulation gene (*WEE1*). Interestingly, *WEE1* and *SA2* are also duplicated in the cyclical parthenogenetic aphid *Acyrthosiphon pisum* and *Daphnia duplex*, respectively, indicating possible roles in the evolution of reproductive mode. Three of these genes (*SA2*, *SCC2*, and *WEE1*) have one copy displaying gonad-specific expression. All genes, with the exception of *WEE1*, have significantly different nonsynonymous/synonymous ratios between the gene duplicates, indicative of a shift in evolutionary constraints following duplication. These results suggest that stick insects may have evolved genes with novel functions in gamete production by gene duplication.

Key words: meiotic gene, phylogenetic distribution, gene duplication, gene expression, parthenogenesis, Phasmatodea.

Significance

Many arthropod species have evolved nonsexual reproductive strategies such as parthenogenesis, yet knowledge of the genetic basis of their evolution is limited. We found several well-characterized meiotic genes, generally being single-copy within Arthropoda, that have more than one copy in the genome of the geographical parthenogenetic stick insect *Clitarchus hookeri*. These paralogs have relatively high level of sequence divergence and together with varied expression levels indicate potential functional divergence. These results establish a basis for further understanding the evolution of different reproductive strategies in these insects.

Introduction

Sexual reproduction is the predominant reproductive strategy in animals, yet many animal groups have repeatedly evolved alternative strategies such as parthenogenesis (obligate and facultative) and the related processes of androgenesis (Schwander and Oldroyd 2016) and gynogenesis (Schlupp 2005). This is particularly true of arthropods, which utilize all

the above strategies (Vershinina and Kuznetsova 2016). However, little is known about the genomic basis of meiosis in lineages that have repeatedly evolved alternative reproductive strategies. Gene family expansion and diversification have long been regarded as sources of evolutionary novelty (Ohno 1970; Lynch and Conery 2000), but their role in the evolution of alternative reproductive strategies is not well understood. Most gene duplicates likely lose function and deteriorate. However, some duplicates may be maintained and ultimately gain new functions, replace some of the functions of other genes, or contribute to dosage compensation (Zhang 2003).

Studies in cyclical parthenogenetic aphids, monogonont rotifers, and the crustacean *Daphnia* have provided evidence of gene duplications in conserved core meiotic genes (King and Serra 1998; Simon et al. 2002; Decaestecker et al. 2009). Notably, most of these duplications are within the genes involved in cell-cycle regulation (Schurko et al. 2009, 2010; Hanson et al. 2013). The WEE1 G2 Checkpoint Kinase (*WEE1*) gene from the pea aphid (*Acyrthosiphon pisum*) and the Cell Division Cycle 20 (*CDC20*) gene from the rotifer (*Brachionus calyciflorus*) are downregulated in asexual compared with sexual forms. These findings suggest additional cell cycle controls during meiosis, and that suppression might alleviate meiotic arrests allowing parthenogenesis to develop (Schurko et al. 2010; Hanson et al. 2013). In addition, genomes of the rotifers (*B. calyciflorus* and *B. manjavacas*) and water flea (*Daphnia duplex*) also have duplicates of genes associated with sister chromatid cohesion (SCC) (*SMC1*, *SMC3*, *SMC6*, *REC8*, *SA*, and *TIM*) and recombination (*RECQ2*). Duplications of some genes involved in RNA silencing (*PIWI* and *AGO*) and DNA replication (*MCM7*) were only detected in the rotifer genomes (Schurko et al. 2009; Hanson et al. 2013). However, whether such duplications are widespread in other animals with a high incidence of reproductive flexibility is yet to be determined.

Few animal groups display such a wide array of reproductive strategies as the insect order Phasmatodea, more commonly known as stick insects. Of the approximately 3,000 species, around 10% are parthenogenetic and scattered in different families (Scali 2009). The females may reproduce by obligate or facultative parthenogenesis (Pijnacker 1966, 1968, 1969; Koch et al. 1972; Pijnacker and Ferwerda 1978; Marescalchi et al. 1991, 1993; Mantovani et al. 1999; Scali et al. 2003; Schwander and Crespi 2009; Buckley and Bradler 2010; Morgan-Richards et al. 2010, 2019; Myers et al. 2013; Alavi et al. 2018; Parker et al. 2019). In addition, other reproductive strategies are known including androgenesis and hybridogenesis (Mantovani and Scali 1992; Scavariello et al. 2017). In Phasmatodea, unfertilized oocytes can organize the first division of embryos without the donation of a centriole from sperm and this is thought to be at least one factor responsible for the repeated evolution of parthenogenesis (Marescalchi et al. 2002). Variations to meiosis include apomixis, automixis, and the modification of meiotic products, which allow the parthenogenetic offspring to achieve a somatic chromosome number (Pijnacker 1969; Marescalchi et al. 1991, 1993; Alavi et al. 2018). However, the molecular basis of these modifications to meiosis in stick insects is largely unknown.

In the New Zealand stick insect fauna, obligate and geographical parthenogenesis is also frequent (Salmon 1991; Jewell and Brock 2002; Buckley et al. 2009, 2010; O'Neill et al. 2009; Morgan-Richards et al. 2010, 2019). *Clitarchus hookeri*, one of the most widespread New Zealand stick insect species, is a geographical parthenogen with sexual populations predominant in the northern and western areas of the North Island and parthenogenetic populations being distributed mostly in the South Island and eastern North Island (Buckley et al. 2010, 2014; Buckley and Bradler 2010; Morgan-Richards et al. 2010, 2019). This species is diploid ($2n = 36$–39), with males lacking one sex chromosome (XO) compared with females (XX) (Parfitt 1980; Morgan-Richards and Trewick 2005). Recent evidence has shown isolated parthenogens can produce sons after mating with males for two generations, which together with an observed deficiency in heterozygotes, suggests that parthenogenesis in *C. hookeri* is automictic where haploid gametes are produced followed by the restoration of the chromosomal number within each gamete (Morgan-Richards et al. 2019).

In this study, we sequenced gonadal transcriptomes from female and male *C. hookeri* using RNA-Seq and identified 97 candidate meiotic genes that have been well-characterized for their roles in meiosis in other systems. We used this gene set to identify gene duplications in candidate *C. hookeri* meiotic genes that are rare or absent in other arthropods, with an emphasis on comparison with species exhibiting flexible reproduction, like stick insects. To determine whether these duplications occurred prior to or following the diversification of Phasmatodea, we also examined these meiotic candidate genes in the genome assembly of the stick insect *Timema cristinae*, sister taxon to all other stick insects (Euphasmatodea) (Whiting et al. 2003; Simon 2019). We then estimated the expression of *C. hookeri* candidate meiotic genes in non-germline and germline tissues and identified those genes with germline-specific expression to test the hypothesis that gene duplication is followed by shifts in gene expression, consistent with the evolution of a novel functionality. We used codon models to detect shifts in substitution patterns between duplicated gene pairs.

Materials and Methods

Sample Collection, Preparation, and RNA Sequencing

Biological replicates of three males and three females were collected from the same population at Totara Park, Auckland,

New Zealand (37° 0.111 S, 174° 55.039 E) for gonadal transcriptome sequencing (supplementary table S1, Supplementary Material online). Female nymphs (third to fifth instars) were collected and then reared until maturation to allow developmental consistency of ovaries for comparison. Specifically, they were reared in separate culture boxes, exposed to room temperature with natural photoperiod in Auckland (35° 52.300 S, 173° 49.290 E). They were fed Manuka (*Leptospermum scoparium*) leaves, which were replaced every 2 days. After maturing, they were checked for egg laying once every day. Each insect was then snap frozen and stored at −80 °C following the first instance of egg laying. Female reproductive tract including approximately 18–20 ovarioles, early developing eggs and oviducts were dissected in ethanol (100%) for RNA extraction. Males were adults when collected, snap frozen and stored at −80 °C directly. Male testicle pairs, appearing to be mature but not degraded, were dissected in ethanol (100%) for RNA extraction. Total RNA extraction and library preparation were performed as described in Wu et al. (2016). The extractions were barcoded individually and then pooled for sequencing on the HISeq2000 platform with three lanes to generate 100 bp PE reads at New Zealand Genomics Limited (NZGL). The pooled data were separated by individual barcode to achieve three individual replicates for each sex.

De Novo Transcriptome Assembly and Quality Assessment

Raw reads were quality assessed with FastQC (Andrews 2010) and then preprocessed as follows: 1) ribosomal RNAs were filtered using SortMeRNA (v2.1) (Kopylova et al. 2012); 2) Illumina universal adapters were screened and trimmed from the 3′ end using CUTADAPT (v1.15) (Martin 2011); 3) 12 bases from the 5′ end, low quality 3′ end (< Q30) and poly-A tail (10 continuous A/T from 3′ end) were trimmed using PRINSEQ (v0.20.4) (Schmieder and Edwards 2011); 4) PRINSEQ was also used to filter reads containing more than one ambiguous base (N) and then maintain pairs with both reads longer than 50 bases; 5) all the maintained read pairs were kept first 68 bases. The resulting reads were then subjected to de novo assembly using TRINITYRNASEQ (v2.6.5) (Grabherr et al. 2011) with applied parameters "–min_contig_length 200; –CPU 8." The assembled transcript set was then cleaned of duplicates, fragments, and alternate transcripts using EvidentialGene (v18-01-2018) (Gilbert 2013), which screens transcripts by converting large, redundant mRNA assembly set to best protein coding sequences. The raw and filtered assemblies were then quality evaluated with Benchmarking Universal Single-Copy Orthologs (BUSCO v3.0.2) (Simao et al. 2015) software (*e*-value: 1e-3), to detect the presence of a core set of 1,066 highly conserved arthropod proteins encoded as single copies.

Gene Identification

Clitarchus hookeri candidate meiotic genes were initially identified by searching protein homologs from other insects against the protein set generated from EvidentialGene. The search set included sequences that were collected from the publications of meiotic amino acid sequences according to the reported sequence IDs from species including *Daphnia pulex* (Schurko et al. 2009), *Acyrthosiphon pisum* (Srinivasan et al. 2010), *Nasonia vitripennis* (Schurko et al. 2010), and *Drosophila melanogaster* (a wings apart-like protein sequence; GenBank ID: NP_001284804.1), as well as gene coding sequences from *Brachionus calyciflorus* and *B. manjavacas* (Hanson et al. 2013). The homologous search was carried out using BLAST (v2.2.30) (McGinnis and Madden 2004) and the BLASTP and TBLASTN (*e*-value < 10) were used with amino acid sequences and gene coding sequences as queries, respectively. The ten best hits of every query sequence were kept and then combined to remove duplicated results. To further determine gene identities, all the resulting *C. hookeri* protein matches were used as query sequences to search against the GenBank *RefSeq* protein database (BLASTP: *e*-value < 10^{-5}; Database was updated on September 28, 2018) and those without the matches to our targeted meiotic proteins were discarded. The resulting *C. hookeri* meiotic proteins were then used to self-search (BLASTP: *e*-value < 10^{-5}) against the same EvidentialGene generated protein set in order to capture genes that were not present as hits from BLAST searches using genes from distantly related species as queries. Similarly, the resulting ten best hits of every query sequence were searched against the GenBank *RefSeq* protein database to determine their gene identities. Finally, to determine whether the putative gene copies are paralogs and not just different alleles, we identified their genomic location by aligning transcripts to the *C. hookeri* genome scaffolds (Wu et al. 2017) using GMAP (v2018-05-30; sequence identities >95%) (Wu and Watanabe 2005).

We searched for orthologs and paralogs of the *C. hookeri* gene duplicates in the stick insect *T. cristinae* genome assembly (v3.0) downloaded from http://nosil-lab.group.shef.ac.uk/?page id=25 (last accessed March 30, 2021) (Riesch et al. 2017). The identified *C. hookeri* meiotic genes were used as a search set to BLAST (TBLASTN, *e*-value < 10) against the *T. cristinae* genome assembly, followed by a prediction of coding regions and amino acids from the scaffolds containing the best blast matches using the online FGENESH+ protein-based gene predictor (Solovyev 2004) (*C. hookeri* protein homologs as comparison and *Tribolium castaneum* as the specific gene-finding parameters). The predicted *T. cristinae* protein sequences were then used as queries to search against the NCBI *RefSeq* database to confirm gene identities. The *C. hookeri* gene copies are predicted as single genes in *T. cristinae* if the two copies had the best hit to one *T. cristinae* homolog.

Phylogenetic Reconstruction and d*N*/d*S* Ratio

Phylogenies were built from gene coding sequences identified in this study and the arthropod species; *Nasonia vitripennis*, *Pediculus humanus*, *Zootemopsis nevadensis*, *Apis mellifera*, *Acyrthosiphon pisum*, *Tribolium castaneum*, *Drosophila melanogaster*, *Aedes aegypti* and *Bombyx mori*, retrieved from InsectBase (Yin et al. 2016) or JGI (*Daphnia pulex* v1.0 Filtered gene models v1.0 and v1.0 Frozen Gene Catalog 7/3/07) (Colbourne et al. 2011). All sequence IDs are provided in supplementary table S2, Supplementary Material online. The alignments were generated using "translation align" with default parameters (MUSCLE alignment) implemented within Geneious (v10.0.9) (Kearse et al. 2012) and then curated on Gblocks online Server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html; last accessed March 30, 2021) (Castresana 2000) using "Codons" and allowing "smaller final blocks" and "gap positions within the final blocks." The degree of nucleotide substitution saturation was assessed using DAMBE (v7.2.152) (Xia 2018). The inferred transition or transversion distances were plotted against the GTR distance. Substitution saturation was considered where the transition distances begin to exceed the transversion distance when plotted against the GTR distance. Maximum-likelihood trees were constructed using PhyML (v2.0) (Guindon et al. 2009) with the best-fit model selected by Jmodeltest (v2.1.10) (Posada 2008; Darriba et al. 2012) under the Akaike Information Criteria (AIC) (Brooks 1989; Posada and Buckley 2004). All the selected best-fit models, calculated gamma values, and proportion of invariable sites for the construction of maximum-likelihood phylogenies are listed in supplementary table S2, Supplementary Material online. Bootstrap support values were calculated by nonparametric bootstrap analysis ($n = 1{,}000$ bootstrap pseudoreplicates). The resulting phylogenies were rendered using FigTREE (v1.4.3) (Rambaut 2009) and were drawn with *Daphnia pulex* as the outgroup if possible.

To infer differences in selective patterns between *C. hookeri* duplicates, d*N*/d*S* ratios were calculated using branch models within the CODEML package of PAML (v4.5) (Yang 2007). Each analysis had both *C. hookeri* duplicates assigned an independent d*N*/d*S* ratio with respect to the rest of the phylogeny. Likelihood ratio tests were conducted between the model allowing for independent ratios, and the associated null model that does not allow for independent ratios. This enables the detection of shifts in the pattern of selection between the two *C. hookeri* duplicates and the background pattern over the rest of the tree (Yang 2007). *HOP1* was not included in the phylogenetic analysis due to a lack of homologous sequences from other sampled arthropod genome assemblies.

Read Quantification and Differential Expression Analysis

Transcript quantification was carried out using "salmon quant" from Salmon (v0.9.1) (Patro et al. 2017) with parameters "-l A -p 8 –gcBias." The cleaned short reads were mapped to the *C. hookeri* transcripts using the Salmon-implemented mapping procedure. Differential expression (DE) analyses were performed in R (v3.5.2) (Ihaka and Gentleman 1996) using the DESeq2 Bioconductor package (Love et al. 2014). This program takes read counts to estimate sample size factors, followed by estimating dispersions by expected mean values from the maximum likelihood estimate of log2 fold changes, and finally fits a negative binomial distribution. The transcripts with an adjusted *P* value less than 0.05 and a minimum fold change (FC) of 2 were reported as significantly differentially expressed. The volcano plot was generated using an R package EnhancedVolcano (Blighe 2018).

Estimation of Gonad-Specific Expression

To infer meiosis-specific expression, four RNA-Seq data sets derived from non-gonadal tissues (antennae, head and prothorax and leg, midgut, and male terminalia) obtained from our previous study (Wu et al. 2016) were aligned to the transcriptome assembly. These were then used to generate transcript per million (TPM) values using Salmon. All the raw data sets were quality re-evaluated and trimmed due to different use of these data sets in this study. The antennae, midgut, and male terminalia reads had duplicated read pairs removed and then had 15 bases trimmed from the 5′ end using FastUniq (Xu et al. 2012) and PRINSEQ, respectively. The reads derived from the head, prothorax, and legs had duplicated pairs removed using FastUniq. Reads were then trimmed using PRINSEQ at the low-quality 3′ ends and filtered for low quality sequences, resulting in lengths of at least 50 bases of cleaned read pairs. A transcript was considered a candidate for gonad-specific expression if it had a TPM value of less than one in all the non-gonad samples (supplementary table S3, Supplementary Material online).

Results

De Novo Assembly and Assessment

A total of 271,860,387 read pairs (supplementary table S1, Supplementary Material online) were used to generate a de novo assembly of the *C. hookeri* gonadal transcriptome. The initial TRINITY assembly was then filtered by EvidentialGene with a comparison shown in table 1. Approximately one-third of the TRINITY transcripts were maintained after filtering with EvidentialGene. BUSCO results show the EvidentialGene processed transcript set had a significant reduction in duplicated sequences, from 66.9% to 2.6%, whereas the estimation of complete proteins remained similar. Therefore, we used the EvidentialGene filtered assembly as the gene pool to search for candidate meiotic genes.

Meiotic Gene Inventory and Expression

We compiled a meiotic core gene list from previous studies on several arthropod species that can switch between sexual and

Table 1
Comparison of the TRINITY and EvidentialGene Filtered Assemblies

	Contig No.	Assembly Size (Mb)	N50 (bp)	GC Content (%)	Contigs Longer than 1 kb (%)	Contig No. (>10 kb)	BUSCO Complete Proteins (%)	BUSCO Duplicated Proteins (%)
TRINITY assembly	359,181	309.15	1,767	40.77	22.4	437	97.9	66.9
EvidentialGene assembly	109,660	99.99	1,685	40.67	25.7	201	96.7	2.6

asexual forms (detailed in Materials and Methods) and used these protein or nucleotide coding sequences to search for *C. hookeri* homologs in the gonadal transcriptome assembly generated in this study. We then filtered the homologs down to a final set of 101 candidate meiotic genes following a search against the NCBI RefSeq database. *Clitarchus hookeri* genes that were homologous to one of the core meiotic genes from previous studies were inferred to be candidate meiosis genes if they were expressed in gonadal tissue (testis and ovarian), but not in the non-gonadal tissues of antennae, head, prothorax, leg, and midgut (Wu et al. 2016). This resulted in 36 candidate meiosis-specific genes. Fifteen of these genes were from the meiotic function catalog of recombination; whereas, seven, five, two, and seven were from SCC, DNA replication, RNA silencing, and cell-cycle regulation catalogs, respectively (supplementary table S3, Supplementary Material online). However, we note that these genes may be expressed in other tissues that we have not sampled or different life stages.

We found there were multiple transcripts annotated as the same gene (*AGO1*, *APC2*, *MSH3*, and *RAD50*) (supplementary table S3, Supplementary Material online). Most of these transcripts were assembled from the same TRINITY read clusters and mapped to the same genomic locations, further indicating they may represent alternatively spliced variants or simply mis-assemblies. We also found that some genes were present on the same genomic scaffold. For example, *HOP2* and *RAD21* were located on scaffold5498, whereas *APC5* and *RECQ4* are on scaffold1732, with 11 exons of *APC5* also distributed on scaffold1101. In addition, there were 21 genes that were split across two scaffolds.

Differential Gene Expression

We compared the transcriptome-wide patterns of DE between females and males. Principle component analysis (PCA) shows that the gene expression patterns from the two tissues are clearly separated with 98% of the variance explained by PC1 (as fig. 1*A*). Compared with male samples, the three females have expression patterns that are much more dispersed even though they were reared in the laboratory and sampled at the same developmental stage (after the first egg was laid) for organ harvest. There were 49 meiotic candidates showing DE, all of which had much higher abundance of transcripts in testis (as fig. 1*B*). Among all the meiotic genes, nearly half of the DE genes (23) also show gonad-specific expression, whereas only 13 non-DE genes (22.4%) were detected with gonad-specific expression.

Meiotic Gene Duplications

We focused on five candidate meiotic genes that showed lineage-specific expansion in either *C. hookeri* or in both *C. hookeri* and *T. cristanae*. Two of these genes are involved in SCC. *SCC2* was the only gene found with two copies in both stick insect species. *SA2* has two copies in *D. melanogaster*, five copies in *Daphnia pulex* and two copies in *C. hookeri.* The chromosomal recombination gene *HOP1*, which can be absent in other assembled insect genomes (Ramesh et al. 2005), has two copies in *C. hookeri*. *AGO2* from the RNA silencing and *WEE1* from cell-cycle regulatory categories were both found with two copies in *C. hookeri*, with these two genes also having rare double gene paralogues in other insects (*AGO2* in *N. vitripennis* and *T. castaneum*; *WEE1* in *N. vitripennis* and *A. pisum*). Phylogenetic reconstructions of *SA2*, *SCC2*, *WEE1*, and *AGO2* are given in figure 2. The *SA2*, *AGO2*, and *WEE1* trees indicate that the two *C. hookeri* duplicates are sister lineages of each other; whereas the *SCC2* duplications, identified from both *C. hookeri* and *T. cristinae*, do not group by species. We were unable to reconstruct the *HOP1* phylogeny due to its absence in most of other taxa (presence in *T. castaneum* and *Z. nevadensis*). However, the two identified copies in *C. hookeri* are much more similar to each other in terms of amino sequence identity (45.1%) compared with sequences from other arthropod genomes (between 22.2% and 43.3%), suggestive of a duplication within Phasmatodea.

None of these stick insect gene copy pairs are arranged tandemly, as assessed by inspection of genomic scaffolds. The *C. hookeri* gene copy pairs exhibited varying numbers of exons and d*N*/d*S* ratios compared with paralogs sequences (table 2). The saturation plots (supplementary fig. S2, Supplementary Material online) did show a degree of saturation in all genes and more so at higher levels of overall sequence divergence. However, we are focusing on the dN and dS rates estimated on the *C. hookeri* tip branches, which are less effected by saturation. To test for differences in selective pressures between the duplicated copies, branch tests were carried out by labelling the two *C. hookeri* gene copies as independent foreground branches. This allows both copies to have independent dN and dS rates from each other, and the rest of the phylogeny. All the genes tested except for *WEE1* had significant likelihood ratios indicating different

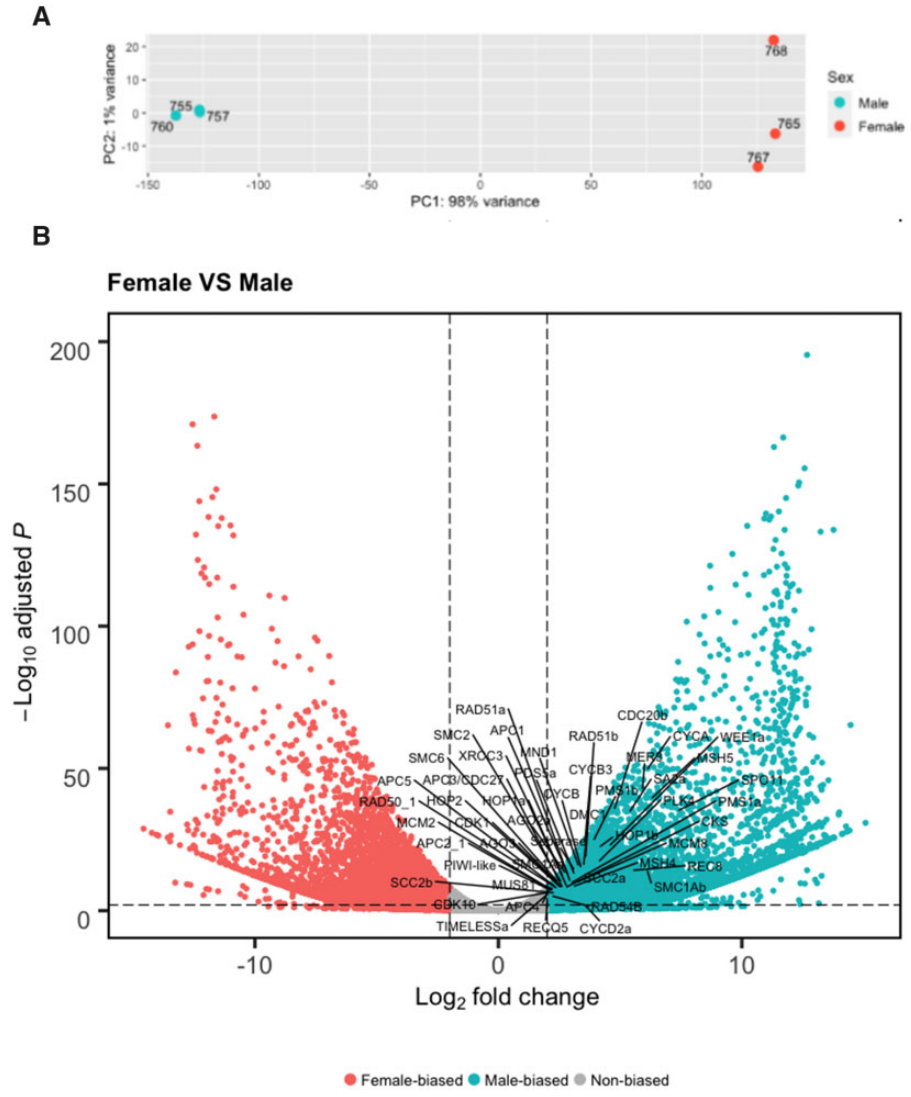

Fig. 1.—Gene DE analysis. (*A*) PCA analysis of overall gene expression per sample. (*B*) Volcano plot showing differential expressed genes between sexes. All the meiotic genes that were differentially expressed are pointed out with gene names. The two dashed vertical lines indicate log2 FC of −2 (left) and 2 (right). The dashed horizontal line indicates adjusted *P* value of 0.05.

codon substitution dynamics between the duplicated gene pairs and the rest of the phylogeny. However, in all cases, the omega values were all less than zero, indicative of overall purifying selection.

Differential Expression between Gene Copies

Aligning short reads from non-gonadal tissues to all of the gene duplicates indicated that the *a* copies of the *SA2*, *SCC2*, and *WEE1* genes are all specifically expressed in the gonads. On the other hand, both copies of *HOP1* and neither of the *AGO2* copies display gonad-specific expression. For each gene, the expression patterns of the two copies vary between females and males (as fig. 3). The gonad-specific *a* copies from *SA2*, *SCC2*, and *WEE1* had much higher expression levels in male than female gonads, whereas the *b* copies had lower expression differences between the two sexes. The two gonad-specific copies of *HOP1* had higher expression in males. The *HOP1a* copy was expressed at a higher level than *b* in females, whereas it showed the opposite pattern in males. The non-gonad-specific copies of *AGO2* showed higher levels of expression of *a* compared with *b* in both sexes and the expression of *b* showed a higher variance among the sample replicates. In addition, *AGO2a* was expressed at a much higher level in males compared with females.

Discussion

The wide array of alternative reproductive strategies in stick insects suggests that meiosis has been modified many times,

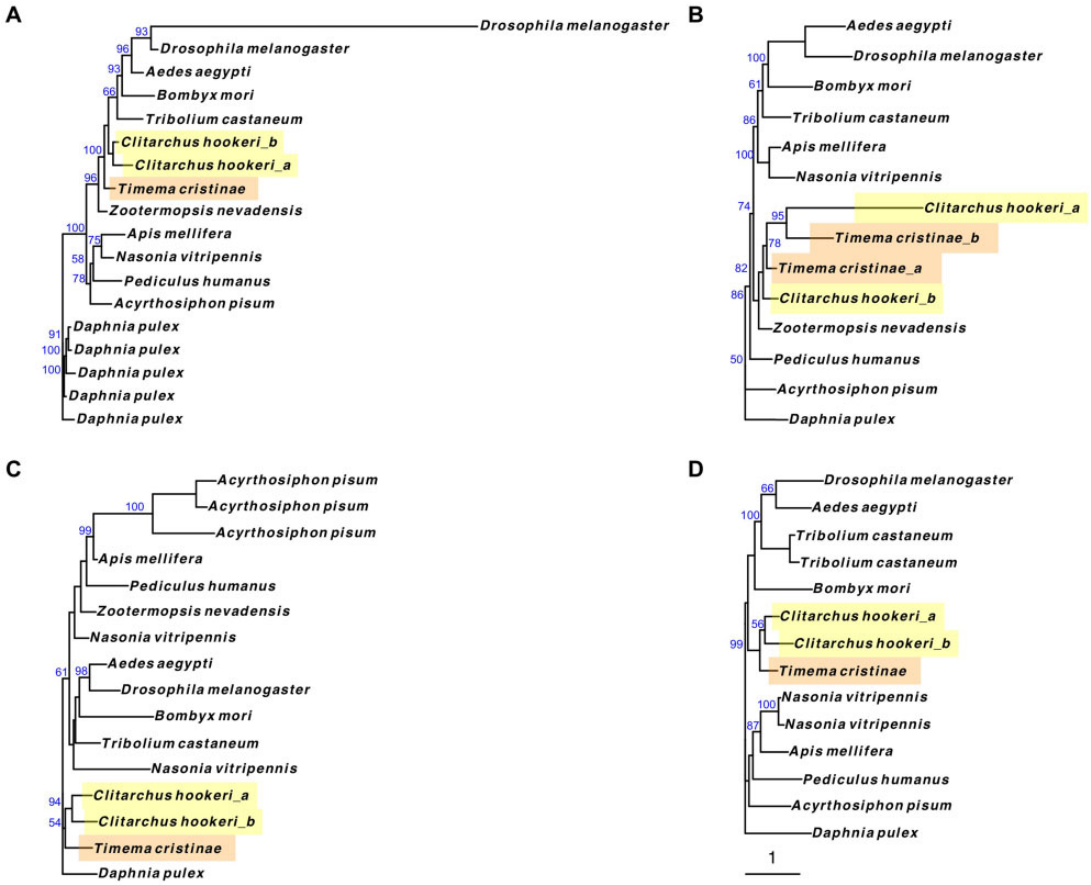

Fig. 2.—Phylogenies of (*A*) *SA2*, (*B*) *SCC2*, (*C*) *WEE1*, and (*D*) *AGO2*. The two stick insect species are highlighted with yellow (*Clitarchus hookeri*) and orange (*Timema cristine*). The duplicated genes of the two stick insect genes are indicated with the copies "*a*" and "*b*" and duplicates in other species are shown with the same species names as they were not given numbers or letters for differentiation. The scale of "1" is applied to all four trees.

Table 2

Duplications of Candidate Meiotic Genes from the *Clitarchus hookeri* Genome

Gene	Copies	Transcript ID	Protein Length (aa)	Amino Acid Identities[#] (%)	Genomic Scaffold ID	Exon Number	ω (d*N*/d*S*) Ratio
SA2	*a*	TRINITY_DN59275_c4_g1_i2	1,107	63	scaffold2017_size403015	20	0.1726
	b	TRINITY_DN59744_c3_g2_i6	1,190		scaffold96_size1435301	6	0.00877
SCC2	*a*	TRINITY_DN54282_c2_g1_i2	1,451	23	scaffold2590_size338625	1	0.23236
	b	TRINITY_DN59006_c0_g1_i1	2,181		scaffold8460_size69818	16	0.00616
WEE1	*a*	TRINITY_DN56459_c4_g1_i2	528	52	scaffold1083_size585250	1	0.00115
	*b**	TRINITY_DN52807_c2_g1_i1	520		scaffold204_size1131059, scaffold28031_size7424	1, 1	0.00814
HOP1	*a*	TRINITY_DN58075_c4_g1_i3	573	38	scaffold2018_size403633	8	—
	b	TRINITY_DN58899_c1_g1_i1	661		scaffold2609_size333860	11	—
AGO2	*a**	TRINITY_DN59887_c5_g2_i1	786	44	scaffold5587_size154872, scaffold4972_size179711	1, 1	0.00113
	b	TRINITY_DN53431_c1_g2_i1	608		scaffold548_size808714	3	0.04382

[a]Indicates genes are found to be split across two scaffolds.
[b]Amino acid identities were measured from aligning amino acid sequences of the gene *a* and *b* using MUSCLE alignment implemented within Geneious.

yet the molecular basis underpinning modifications to meiosis are not well understood. In this study, we used *C. hookeri*, a geographical parthenogenetic stick insect, and *T. cristinae*, the sister taxon to all the other stick insects, as model species to investigate meiotic gene duplication, shifts in gene expression and patterns of selection at the codon level. In *C. hookeri*

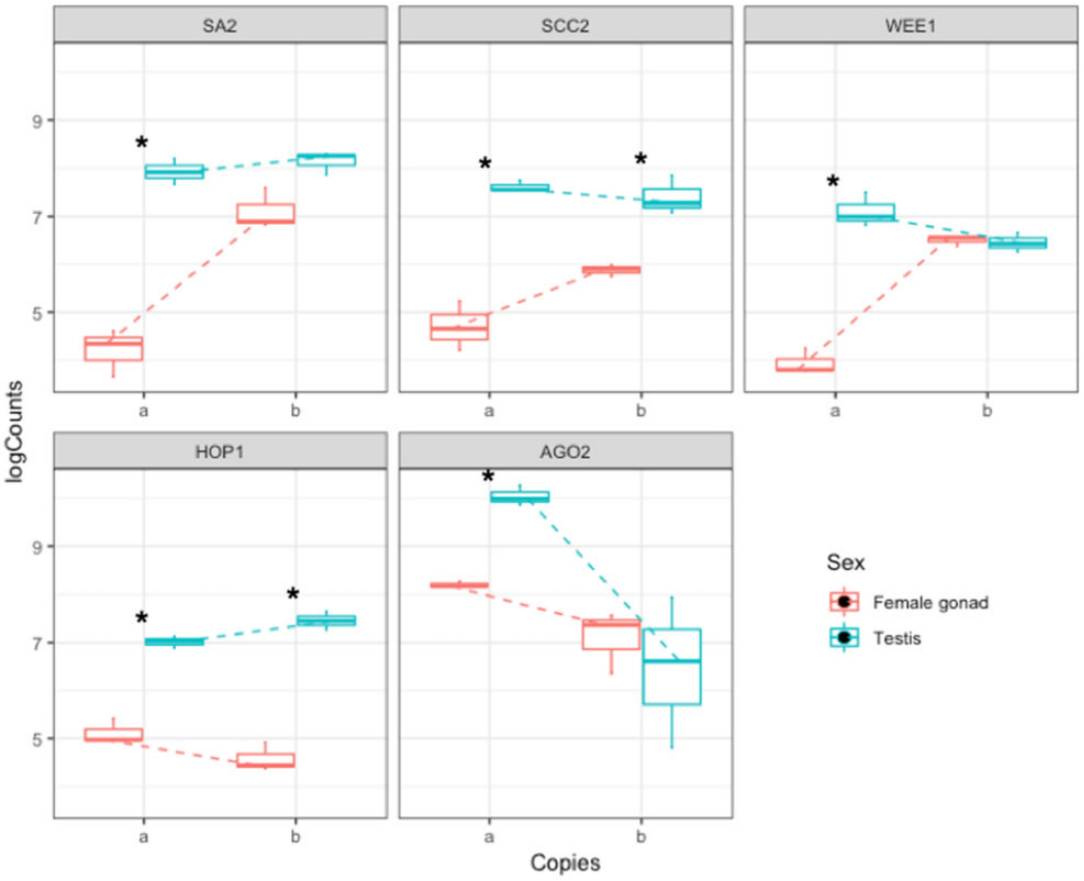

Fig. 3.—Boxplots showing expression difference of each duplicate pair between sexes.

we identified multiple lineage-specific gene duplicates together with varied expression and evolutionary rates between each pair of duplicate genes. This finding contrasts with *T. cristinae* where only a single duplication event was observed (Bradler et al. 2015).

Meiotic Gene Inventory and Expression

All the meiosis-associated genes found with expressed homologs in the *C. hookeri* transcriptome also have *T. cristinae* gene orthologs, suggesting a high degree of conservation in these genes between the two stick insect genomes. Given that *Timema* is the sister taxon to all other stick insects (Whiting et al. 2003), it is also possible that this meiotic gene repertoire is conserved across the Phasmatodea. The expression of a complete set of the core meiotic recombination gene homologs in the *C. hookeri* reproductive organs is consistent with a role in sexual reproduction and *T. cristinae* is likely to utilize the same meiotic recombination machinery. Although some genes from this inventory are absent in other sexual and cyclical parthenogenetic arthropods (Ramesh et al. 2005; Schurko et al. 2009, 2010; Hanson et al. 2013; Tvedte et al. 2017), they seem to be maintained in at least these two stick insect species.

The comparison between gonadal and a wide range of non-gonadal tissues identified 36 predicted meiotic gene candidates with gonad-specific expression, consistent with their previously hypothesized role in meiosis. As expected, many of these genes (15) are involved in recombination-related functions. Similar to many obligate sexual organisms, candidate meiosis-specific genes (genes with gonad-specific expression) from *C. hookeri* also include the genes encoding the cohesion subunit *REC8* (Stoop-Myer and Amon 1999), the synaptonemal complex components *HOP1* and *HOP2* (Leu et al. 1998; Anuradha and Muniyappa 2004), the double-strand break (DSB) initiator *SPO11* (Keeney et al. 1997; Keeney 2008), the mismatch repair protein *MSH4* (Paquis-Flucklinger et al. 1997) and *MSH5* (Snowden et al. 2004), as well as the DSB end resect protein *MRE11* (Johzuka and Ogawa 1995). Although *DMC1* encoding proteins that play a role in DSB strand innovation and displacement have been reported as meiosis specific (Sehorn et al. 2004), in our data they were found to be expressed in both gonad and non-gonad tissues (leg, head, antennae, and midgut), indicating possible functions in mitosis (Maciver et al. 2019). The seven genes involved in cell-cycle regulation that displayed gonad-specific expression may contribute to determining whether the cell cycle is

meiotic rather than mitotic; for example, additional time controls for allowing recombination and the meiosis I to meiosis II transition (Marston and Amon 2004). Comparative analysis of gene expression between female and male gonads revealed that all differentially expressed meiotic genes were male biased and mostly gonad specific. This pattern of higher expression levels of meiotic genes in males matches our expectations. Female gonads only contain a limited number of cells from their ovariolar tips that are undergoing meiosis, compared with testis, that include a far greater percentage of cells in the process of spermatogenesis.

Lineage-Specific Gene Duplication and Expression

A previous study assembled the genome of *C. hookeri* to a size of approximately 4.2 Gb, roughly four times that of *T. cristinae* (Wu et al. 2017). The difference in genome size can largely be attributed to a dramatically higher repetitive element content and longer introns in *C. hookeri* (Wu et al. 2017) relative to *T. cristinae*. *Clitarchus hookeri* also has numerous duplicated genes relative to *T. cristinae* including those encoding phosphoglucose isomerase (Dunning et al. 2013) and cellulase (Shelomi et al. 2016; Wu et al. 2016). In this study, we found four meiotic genes with lineage-specific gene duplications in *C. hookeri*, but not in *T. cristinae*, evidence of further gene family expansion in *C. hookeri* relative to *T. cristinae*. It is also possible that there are more meiotic gene duplications in the *C. hookeri* genome, that were not detected from our current transcriptome due to, for example, low levels of gene expression. However, we did not perform a search against the available *C. hookeri* genome assembly because: 1) genes identified from the genome assembly can be pseudogenes or expressed in other tissues that are not associated with reproduction, 2) the genomic scaffolds are still highly fragmented with over 2% of bases being gaps (21 meiotic transcripts were split across multiple genomic scaffolds), and 3) more complete genes are likely to be absent in the genome assembly than the transcriptome (91.6% of BUSCO proteins are present in the genome assembly whereas 96.7% are present in the transcriptome assembled in this study). However, it is possible that apparent gene duplicates identified from our short read assembled transcriptomes are alleles or isoforms rather than true duplicates. We suggest this is not likely to be the case for our proposed duplicate pairs because each gene copy was present on separate genomic scaffolds, varied in exon number and were highly diverged from each other on the basis of amino acid identities (less than 64%, table 2) and phylogenetic analysis (as fig. 2). The duplicates from *SA2* and *WEE1* also have expression bias measured between gonadal and non-gonadal tissues. The genes *SA2a* and *WEE1a* were found to be expressed specifically in gonadal tissues, whereas the copy "*b*" of these two genes also had expression in non-gonadal tissues (supplementary table S3, Supplementary Material online). For each gene, the expression patterns of the two copies varied between females and males (as fig. 3).

Sister Chromatid Cohesion

SCC is a process required for connecting the newly formed sister chromatids from DNA duplication and lasts from S phase to anaphase onset during mitosis and meiosis (Peters and Nishiyama 2012). *SA2* is a subunit of the SCC complex (cohesin) that forms a ring structure together with *SMC3* and *RAD21* (mitosis)/REC8 (meiosis) to bind sister chromosomes (Peters and Nishiyama 2012). In *Drosophila*, *Stromalin* (*SNM*) is the paralogous duplicate of *SA2*, and encodes proteins present along the lengths of homologous chromosomes that are essential for homologous pairing and chromosome segregation in male meiosis (Thomas et al. 2005). *SNM* has a faster rate of evolution than *SA* (Thomas et al. 2005; Beekman 2013). Similarly, the genome of *C. hookeri* also harbors two copies of *SA2* and the *a* copy has gonad-specific expression along with a longer branch length compared with the more widely expressed *b* copy. Unlike *Drosophila*, the *a* copy also shows expression in the female gonads. Interestingly, this gene has five copies in the genome of the cyclical parthenogenetic *Daphnia pulex* (Schurko et al. 2009, 2010).

SCC2 is one of the components of another protein complex, which is responsible for loading cohesin onto the chromosomes (Ciosk et al. 2000). This complex also interacts with cohesin and other proteins, such as *PDS5*, *ESPL1* (*Separase*) and *WAPL*, to stabilize the ring structure and enable cohesion success and effective release of the cohesin from chromosomes (Panizza et al. 2000; Peters and Nishiyama 2012). *SCC2* is a highly conserved gene, with homologs found across a wide range of eukaryote species (Seitz et al. 1996; Furuya et al. 1998; Rollins et al. 1999). In meiosis, proteins encoded by this gene, in addition to being involved in recruiting cohesins, are also involved in synaptonemal assembly and maintenance (Gause et al. 2008). The *SCC2* gene was duplicated in both stick insect genomes before the two species diverged. Similar to *SA2*, the *C. hookeri a* copy is expressed only in gonads and has a longer branch length than the universally expressed *b* copy. These results indicate the meiotic functions of this gene might have shifted a long time ago and whether the *b* copy still maintains a role in meiosis requires further investigation. The gonad-specific copies *SA2a* and *SCC2a* may have been under less stringent selective constraints due to tissue-limited expression and subsequently accumulated more substitutions compared with the widely expressed *b* copy.

Synaptomal Complex Component

HOP1 was found to have an extra copy in the *C. hookeri* genome relative to *T. cristinae*. In yeast, this gene is specific to meiosis, acting as a component of the synaptonemal complex, and plays a significant role in chromosomal pairing as

well as participating in inter-homolog recombination and crossing over (Hollingsworth and Byers 1989; Hollingsworth et al. 1990; Anuradha and Muniyappa 2004; Anuradha et al. 2005). Interestingly, this gene, although generally conserved in most other eukaryotes is missing in some insect lineages, such as the sexually reproducing *Drosophila* and *Anopheles* (Ramesh et al. 2005). In our study, the survey of *HOP1* in *Daphnia pulex* and 11 insect species demonstrates that it is only present in the three Polyneoptera species (*Z. nevadensis: XM_022072157.1*, *T. cristinae*, and *C. hookeri*) and the beetle *Tribolium castaneum* (*TCOGS2: TC000115*). The expression of the two *C. hookeri* copies appears to be gonad specific, but the expression of *a* is higher than *b* in the females, whereas the opposite pattern is apparent in males, indicating their involvement in sex-specific processes, possibly during meiotic recombination. It is interesting that in arthropods, *HOP1* can be missing in both sexual (Ramesh et al. 2005; Srinivasan et al. 2010; Hanson et al. 2013) and cyclical parthenogenetic species (*A. pisum* and *D. duplex* from this study), indicating its presence does not correlate well with reproductive mode. However, our discovery of a *HOP1* duplication in arthropods raises interesting questions about its function and evolution.

RNA Silencing and Cell-Cycle Regulation

Both stick insect genomes express all the genes included in the categories of RNA silencing and cell-cycle regulation from our a priori gene list. *Clitarchus hookeri* has two duplication events of the genes *AGOB* ($n = 2$) and *WEE1* ($n = 2$), whereas only one copy of each was found in the *T. cristinae* genome. Argonaute proteins form the functional core of the RNA-induced silencing complexes, which mediate RNA silencing in eukaryotes. In mammals, *AGO1-4* forms the complex that mediates RNA cleavage targeted by micro-RNAs (miRNAs) and small-interfering RNAs (siRNAs) (Liu et al. 2004; Meister et al. 2004), some of which are also involved in the biogenesis of miRNAs and siRNAs (Yang and Lai 2010; Meister 2013). Inactive *AGO2* in mouse results in aberrant meiotic maturation and defects in spindle formation and chromosome alignment during oogenesis (Stein et al. 2015). *MEL1*, an *AGO* homolog in rice (*Oryza sativa*) regulates cell division of premeiotic germ cells, and is involved in the proper modification of meiotic chromosomes and meiotic progression (Nonomura et al. 2007). It is interesting that there are multiple gene duplication events in the argonaute family (*piwi*: $n = 8$; *AGO3*: $n = 2$) from the cyclical parthenogenetic *A. pisum* and that some of the gene members (*piwi3*, *piwi8*, and *AGO3a*) are differentially expressed in ovaries between sexual and asexual forms (Lu et al. 2011; Srinivasan et al. 2014). Multiple copies of *AGOB* have been also reported in *N. vitripennis* ($n = 3$) and the cyclical parthenogenetic *B. calyciorus* ($n = 2$) (Schurko et al. 2010; Hanson et al. 2013). In addition, we found that the *Tribolium castaneum* genome has two copies of *AGOB*. The six members of the Argonaute family that we found in the *C. hookeri* transcriptome have provided candidates to study the evolution of geographic parthenogenesis in this species.

WEE1 is a subunit of the G2/M checkpoint protein complex, which inhibits cyclin-dependent kinase *CDK1* activities through phosphorylation, thereby ensuring mature entry into mitosis and meiosis (Nurse and Thuriaux 1980; Den Haese et al. 1995). It also plays a role in the cell size checkpoint by coordinating cell size and cell cycle progression (Kellogg 2003). In *Drosophila*, proper regulation of the early syncytial cycles of embryogenesis requires inhibitory phosphorylation of *CDK1* by *WEE1* (Price et al. 2000; Stumpff et al. 2004). Interestingly, the expansion of this gene family seems to frequently occur in species that display alternative reproductive modes. The cyclical parthenogenetic pea aphid has three *WEE1* duplicates, and most interestingly, they are all down-regulated in asexual pea aphids compared with sexual forms, suggesting an evolved additional cell cycle checkpoint control that may be associated with reproductive flexibility (Srinivasan et al. 2010). *WEE1* duplicates ($n = 2$) have also been found in the cyclical parthenogenetic rotifer *Brachionus manjavacas* (Hanson et al. 2013). In our study, we found an additional *WEE1* duplication event ($n = 2$) in the *C. hookeri* transcriptome, and so far, duplication of this gene in arthropods has only been seen in these three species displaying reproductive flexibility.

Conclusion

We have identified 101 candidate meiotic genes and their expression patterns in the stick insect *C. hookeri*. Five of these genes were duplicated in *C. hookeri* and 1 in *Timema* that are rarely or never duplicated in the other arthropod genome assemblies we surveyed. Three of the five genes showed gonad-specific expression in one of the duplicates. These duplicates also showed evidence of shifts in substitution pattern between the duplicate pairs but with overall purifying selection. These inferences have shed light on the genes underlying meiosis in stick insects. Future work will be needed to test the function of the candidate genes examined here. Also, an examination of differential gene expression in these candidate genes between sexual and parthenogenetic populations of *C. hookeri*, may reveal the molecular switches underlying parthenogenesis.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

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Author Contributions

T.R.B. and R.D.N. conceived and designed the project and advised on analyses. C.W. collected samples and performed de novo assembly, gene identification, and comparative transcriptomic analysis. V.G.T. and C.W. performed RNA extraction. V.G.T. performed PAML analysis. C.W. prepared the manuscript draft. T.R.B. and R.D.N. edited and commented extensively on the draft manuscript. All authors reviewed and approved the final manuscript.

Data Availability

RNA sequencing (RNA-Seq) reads and the de novo transcriptome assembly are deposited in the National Centre for Biotechnology Information (NCBI) under the project PRJNA395945.

Literature Cited

Alavi Y, Rooyen A, Elgar MA, Jones TM, Weeks AR. 2018. Novel microsatellite markers suggest the mechanism of parthenogenesis in *Extatosoma tiaratum* is automixis with terminal fusion. Insect Sci. 25(1):24–7917.

Andrews S. 2010. FastQC: a quality control tool for high throughput sequence data. Available from: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/.

Anuradha S, Muniyappa K. 2004. Meiosis-specific yeast Hop1 protein promotes synapsis of double-stranded DNA helices via the formation of guanine quartets. Nucleic Acids Res. 32(8):2378–2385.

Anuradha S, Tripathi P, Mahajan K, Muniyappa K. 2005. Meiosis-specific yeast Hop1 protein promotes pairing of double-stranded DNA helices via G/C isochores. Biochem Biophys Res Commun. 336(3):934–941.

Beekman DJ. 2013. The evolution and expression of *Drosophila meiosis* genes [PhD thesis]. University of Iowa.

Blighe K. 2018. EnhancedVolcano: publication-ready volcano plots with enhanced colouring and labeling. Available from: https://github.com/kevinblighe

Bradler S, Cliquennois N, Buckley TR. 2015. Single origin of the Mascarene stick insects: ancient radiation on sunken islands? BMC Evol Biol. 15:196.

Brooks DG. 1989. Akaike information criterion statistics. Technometrics 31(2):270–271.

Buckley TR, Bradler S. 2010. *Tepakiphasma ngatikuri*, a new genus and species of stick insect (Phasmatodea) from the Far North of New Zealand. N Z Entomol. 33(1):118–126.

Buckley TR, Marske KA, Attanayake D. 2009. Identifying glacial refugia in a geographic parthenogen using palaeoclimate modelling and phylogeography: the New Zealand stick insect *Argosarchus horridus* (White). Mol Ecol. 18(22):4650–4663.

Buckley TR, Marske K, Attanayake D. 2010. Phylogeography and ecological niche modelling of the New Zealand stick insect *Clitarchus hookeri* (White) support survival in multiple coastal refugia. Jf Biogeogr. 37(4):682–695.

Buckley TR, Myers SS, Bradler S. 2014. Revision of the stick insect genus *Clitarchus* Stal (Phasmatodea: phasmatidae): new synonymies and two new species from northern New Zealand. Zootaxa 3900(4):451–482.

Castresana J. 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol. 17(4):540–552.

Ciosk R, et al. 2000. Cohesin's binding to chromosomes depends on a separate complex consisting of Scc2 and Scc4 proteins. Mol Cell. 5(2):243–254.

Colbourne JK, et al. 2011. The ecoresponsive genome of Daphnia pulex. Science 331(6017):555–561.

Darriba D, Taboada GL, Doallo R, Posada D. 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat Methods. 9(8):772.

Decaestecker E, De Meester L, Mergeay J. 2009. Cyclical parthenogenesis in *Daphnia*: sexual versus asexual reproduction. In: Schön, I, Martens, K, and Dijk, P, editors. Lost sex: the evolutionary biology of parthenogenesis. Netherlands: Springer Nature. p. 295–316.

Den Haese G, Walworth N, Carr A, Gould K. 1995. The Wee1 protein kinase regulates T14 phosphorylation of fission yeast Cdc2. Mol Biol Cell. 6(4):371–385.

Dunning LT, et al. 2013. Positive selection in glycolysis among Australasian stick insects. BMC Evol Biol. 13:215.

Furuya K, Takahashi K, Yanagida M. 1998. Faithful anaphase is ensured by Mis4, a sister chromatid cohesion molecule required in S phase and not destroyed in G1 phase. Genes Dev. 12(21):3408–3418.

Gause M, et al. 2008. Functional links between *Drosophila* Nipped-B and cohesin in somatic and meiotic cells. Chromosoma 117(1):51–66.

Gilbert D. 2013. Gene-omes built from mRNA seq not genome DNA. Paper presented at the 7th Annual Arthropod Genomics Symposium, Notre Dame. Available from: http://arthropods.eugenes.org/EvidentialGene/about/EvigeneRNA2013poster.pdf

Grabherr MG, et al. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol. 29(7):644–652.

Guindon S, Dufayard JF, Hordijk W, Lefort V, Gascuel O. 2009. PhyML: fast and accurate phylogeny reconstruction by maximum likelihood. Infect Genet Evol. 9:384–385.

Hanson SJ, et al. 2013. Inventory and phylogenetic analysis of meiotic genes in monogonont rotifers. J Hered. 104(3):357–370.

Hollingsworth NM, Byers B. 1989. HOP1: a yeast meiotic pairing gene. Genetics 121(3):445–462.

Hollingsworth NM, Goetsch L, Byers B. 1990. The HOP1 gene encodes a meiosis-specific component of yeast chromosomes. Cell 61(1):73–84.

Ihaka R, Gentleman R. 1996. R: a language for data analysis and graphics. J Comput Graph Stat. 5(3):299–314.

Jewell T, Brock PD. 2002. A review of the New Zealand stick insects: new genera and synonymy, keys, and a catalogue. J Orthop Res. 11(2):189–197.

Johzuka K, Ogawa H. 1995. Interaction of Mre11 and Rad50: two proteins required for DNA repair and meiosis-specific double-strand break formation in *Saccharomyces cerevisiae*. Genetics 139(4):1521–1532.

Kearse M, et al. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28(12):1647–1649.

Keeney S. 2008. Spo11 and the formation of DNA double-strand breaks in meiosis. In: Egel R, Lankenau D-H, editors. Recombination and meiosis: crossing-over and disjunction. Berlin/Heidelberg (Germany): Springer. p. 81–123.

Keeney S, Giroux CN, Kleckner N. 1997. Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. Cell 88(3):375–384.

Kellogg DR. 2003. Wee1-dependent mechanisms required for coordination of cell growth and cell division. J Cell Sci. 116(Pt 24):4883–4890.
King CE, Serra M. 1998. Seasonal variation as a determinant of population structure in rotifers reproducing by cyclical parthenogenesis. Hydrobiologia 387:361–372.
Koch P, Pijnacker L, Kreke J. 1972. DNA reduplication during meiotic prophase in the oocytes of *Carausius morosus* Br. (Insecta, Cheleutoptera). Chromosoma 36(3):313–321.
Kopylova E, Noe L, Touzet H. 2012. SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. Bioinformatics 28(24):3211–3217.
Leu J-Y, Chua PR, Roeder GS. 1998. The meiosis-specific Hop2 protein of *S. cerevisiae* ensures synapsis between homologous chromosomes. Cell 94(3):375–386.
Liu J, et al. 2004. Argonaute2 is the catalytic engine of mammalian RNAi. Science 305(5689):1437–1441.
Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15(12):550.
Lu HL, et al. 2011. Expansion of genes encoding piRNA-associated argonaute proteins in the pea aphid: diversification of expression profiles in different plastic morphs. PLoS One 6(12):e28051.
Lynch M, Conery JS. 2000. The evolutionary fate and consequences of duplicate genes. Science 290(5494):1151–1155.
Maciver SK, Koutsogiannis Z, de Obeso Fernández del Valle A. 2019. 'Meiotic genes' are constitutively expressed in an asexual amoeba and are not necessarily involved in sexual reproduction. Biol Lett. 15(3):20180871.
Mantovani B, Passamonti M, Scali V. 1999. Genomic evolution in parental and hybrid taxa of the genus *Bacillus* (Insecta Phasmatodea). Italian J Zool. 66(3):265–272.
Mantovani B, Scali V. 1992. Hybridogenesis and androgenesis in the stick-insect *Bacillus rossius-Grandii benazzii* (Insecta, Phasmatodea). Evolution 46(3):783–796.
Marescalchi O, Pijnacker L, Scali V. 1991. Cytology of parthenogenesis in *Bacillus whitei* and *Bacillus lynceorum* (Insecta Phasmatodea). Invert Reprod Dev. 20(1):75–81.
Marescalchi O, Pijnacker L, Scali V. 1993. Automictic parthenogenesis and its genetic consequence in *Bacillus atticus atticus* (Insecta Phasmatodea). Invert Reprod Dev. 24(1):7–12.
Marescalchi O, Zauli C, Scali V. 2002. Centrosome dynamics and inheritance in related sexual and parthenogenetic *Bacillus* (Insecta phasmatodea). Mol Reprod Dev. 63(1):89–95.
Marston AL, Amon A. 2004. Meiosis: cell-cycle controls shuffle and deal. Nat Rev Mol Cell Biol. 5(12):983–997.
Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. Embnet J. 17(1):10.
McGinnis S, Madden TL. 2004. BLAST: at the core of a powerful and diverse set of sequence analysis tools. Nucleic Acids Res. 32(Web Server issue):W20–W25.
Meister G. 2013. Argonaute proteins: functional insights and emerging roles. Nat Rev Genet. 14(7):447–459.
Meister G, et al. 2004. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Mol Cell. 15(2):185–197.
Morgan-Richards M, Langton-Myers SS, Trewick SA. 2019. Loss and gain of sexual reproduction in the same stick insect. Mol Ecol. 28(17):3929–3941.
Morgan-Richards M, Trewick SA. 2005. Hybrid origin of a parthenogenetic genus? Mol Ecol. 14(7):2133–2142.
Morgan-Richards M, Trewick SA, Stringer IA. 2010. Geographic parthenogenesis and the common tea-tree stick insect of New Zealand. Mol Ecol. 19(6):1227–1238.
Myers SS, Trewick SA, Morgan-Richards M. 2013. Multiple lines of evidence suggest mosaic polyploidy in the hybrid parthenogenetic stick insect lineage *Acanthoxyla*. Insect Conserv Divers. 6(4):537–548.
Nonomura K-I, et al. 2007. A germ cell-specific gene of the ARGONAUTE family is essential for the progression of premeiotic mitosis and meiosis during sporogenesis in rice. Plant Cell. 19(8):2583–2594.
Nurse P, Thuriaux P. 1980. Regulatory genes controlling mitosis in the fission yeast *Schizosaccharomyces pombe*. Genetics 96(3):627–637.
O'Neill SB, Buckley TR, Jewell TR, Ritchie PA. 2009. Phylogeographic history of the New Zealand stick insect *Niveaphasma annulata* (Phasmatodea) estimated from mitochondrial and nuclear loci. Mol Phylogenet Evol. 53(2):523–536.
Ohno S. 1970. Evolution by gene duplication. Berlin/Heidelberg (Germany): Springer.
Panizza S, Tanaka T, Hochwagen A, Eisenhaber F, Nasmyth K. 2000. Pds5 cooperates with cohesin in maintaining sister chromatid cohesion. Curr Biol. 10(24):1557–1564.
Paquis-Flucklinger V, et al. 1997. Cloning and expression analysis of a meiosis-specific MutS homolog: the human MSH4 gene. Genomics 44(2):188–194.
Parfitt RG. 1980. Cytology and feulgen – DNA microdensitometry of New Zealand stick insects (Phasmatodea: Phasmatidae) [MSc thesis]. Victoria University of Wellington.
Parker DJ, et al. 2019. Sex-biased gene expression is repeatedly masculinized in asexual females. Nat Commun. 10(1):4638.
Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C. 2017. Salmon provides fast and bias-aware quantification of transcript expression. Nat Methods. 14(4):417–419.
Peters J-M, Nishiyama T. 2012. Sister chromatid cohesion. Cold Spring Harb Perspect Biol. 4(11):a011130.
Pijnacker L. 1966. The maturation divisions of the parthenogenetic stick insect *Carausius morosus* Br. (Orthoptera, Phasmidae). Chromosoma 19(1):99–112.
Pijnacker L. 1968. Oogenesis in the parthenogenetic stick insect *Sipyloidea sipylus* Westwood (Orthoptera, Phasmidae). Genetica 38(4):504–515.
Pijnacker L. 1969. Automictic parthenogenesis in the stick insect *Bacillus rossius* Rossi (Cheleutoptera, phasmidae). Genetica 40(1):393–399.
Pijnacker L, Ferwerda M. 1978. Additional chromosome duplication in female meiotic prophase of *Sipyloidea sipylus* Westwood (Insecta, Phasmida), and its absence in male meiosis. Experientia 34(12):1558–1560.
Posada D. 2008. jModelTest: phylogenetic model averaging. Mol Biol Evol. 25(7):1253–1256.
Posada D, Buckley TR. 2004. Model selection and model averaging in phylogenetics: advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. Syst Biol. 53(5):793–808.
Price D, Rabinovitch S, O'Farrell PH, Campbell SD. 2000. *Drosophila* wee1 has an essential role in the nuclear divisions of early embryogenesis. Genetics 155(1):159–166.
Rambaut A. 2009. FigTree version 1.3.1. Available from http://tree.bio.ed.ac.uk.
Ramesh MA, Malik SB, Logsdon JM Jr. 2005. A phylogenomic inventory of meiotic genes; evidence for sex in Giardia and an early eukaryotic origin of meiosis. Curr Biol. 15(2):185–191.
Riesch R et al. 2017. Transitions between phases of genomic differentiation during stick-insect speciation. Nat Ecol Evol. 1(4):0082.
Rollins RA, Morcillo P, Dorsett D. 1999. Nipped-B, a *Drosophila* homologue of chromosomal adherins, participates in activation by remote enhancers in the cut and Ultrabithorax genes. Genetics 152(2):577–593.
Salmon JT. 1991. The stick insects of New Zealand. Raupo: New Holland Publishers (NZ) Ltd.
Scali V. 2009. Metasexual stick insects: model pathways to losing sex and bringing it back. In: Schön I, Martens K, Dijk P, editors. Lost sex: the evolutionary biology of parthenogenesis. Netherlands: Springer Nature. p. 317–345.

Scali V, Passamonti M, Marescalchi O, Mantovani B. 2003. Linkage between sexual and asexual lineages: genome evolution in *Bacillus* stick insects. Biol J Linnaean Soc. 79(1):137–150.
Scavariello C, Luchetti A, Martoni F, Bonandin L, Mantovani B. 2017. Hybridogenesis and a potential case of R2 non-LTR retrotransposon horizontal transmission in *Bacillus* stick insects (Insecta Phasmida). Sci Rep. 7:41946.
Schlupp I. 2005. The evolutionary ecology of gynogenesis. Annu Rev Ecol Evol Syst. 36(1):399–417.
Schmieder R, Edwards R. 2011. Quality control and preprocessing of metagenomic datasets. Bioinformatics 27(6):863–864.
Schurko AM, Logsdon JM, Eads BD. 2009. Meiosis genes in *Daphnia pulex* and the role of parthenogenesis in genome evolution. BMC Evol Biol. 9:78.
Schurko AM, Mazur DJ, Logsdon JM Jr. 2010. Inventory and phylogenomic distribution of meiotic genes in *Nasonia vitripennis* and among diverse arthropods. Insect Mol Biol. 19:165–180.
Schwander T, Crespi BJ. 2009. Multiple direct transitions from sexual reproduction to apomictic parthenogenesis in *Timema* stick insects. Evolution 63(1):84–103.
Schwander T, Oldroyd BP. 2016. Androgenesis: where males hijack eggs to clone themselves. Philos Trans R Soc Lond B Biol Sci. 371: 1706.
Sehorn MG, Sigurdsson S, Bussen W, Unger VM, Sung P. 2004. Human meiotic recombinase Dmc1 promotes ATP-dependent homologous DNA strand exchange. Nature 429(6990):433–437.
Seitz LC, Tang K, Cummings WJ, Zolan ME. 1996. The rad9 gene of *Coprinus cinereus* encodes a proline-rich protein required for meiotic chromosome condensation and synapsis. Genetics 142(4):1105–1117.
Shelomi M, et al. 2016. Horizontal gene transfer of pectinases from bacteria preceded the diversification of stick and leaf insects. Sci Rep. 6:26388.
Simao FA, Waterhouse RM, Ioannidis P, Kriventseva EV, Zdobnov EM. 2015. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. Bioinformatics 31(19):3210–3212.
Simon S, et al. 2019. Old World and New World Phasmatodea: Phylogenomics resolve the evolutionary history of stick and leaf insects. Front Ecol Evol. 7:345.
Simon J-C, Rispe C, Sunnucks P. 2002. Ecology and evolution of sex in aphids. Trends Ecol Evol. 17(1):34–39.
Snowden T, Acharya S, Butz C, Berardini M, Fishel R. 2004. hMSH4-hMSH5 recognizes Holliday Junctions and forms a meiosis-specific sliding clamp that embraces homologous chromosomes. Mol Cell. 15(3):437–451.
Solovyev V. 2004. Statistical approaches in eukaryotic gene prediction. In: Cannings C, Balding DJ, Bishop M, editors. Handbook of statistical genetics 1. Chichester: Wiley. p. 4.
Srinivasan DG, Abdelhady A, Stern DL. 2014. Gene expression analysis of parthenogenetic embryonic development of the pea aphid, *Acyrthosiphon pisum*, suggests that aphid parthenogenesis evolved from meiotic oogenesis. PLoS One 9(12):e115099.
Srinivasan DG, Fenton B, Jaubert-Possamai S, Jaouannet M. 2010. Analysis of meiosis and cell cycle genes of the facultatively asexual pea aphid, *Acyrthosiphon pisum* (Hemiptera: aphididae). Insect Mol Biol. 19:229–239.
Stein P, et al. 2015. Essential Role for endogenous siRNAs during meiosis in mouse oocytes. PLoS Genet. 11(2):e1005013.
Stoop-Myer C, Amon A. 1999. Meiosis: rec8 is the reason for cohesion. Nat Cell Biol. 1(5):E125–E127.
Stumpff J, Duncan T, Homola E, Campbell SD, Su TT. 2004. *Drosophila* Wee1 kinase regulates Cdk1 and mitotic entry during embryogenesis. Curr Biol. 14(23):2143–2148.
Thomas SE, et al. 2005. Identification of two proteins required for conjunction and regular segregation of achiasmate homologs in Drosophila male meiosis. Cell 123(4):555–568.
Tvedte ES, Forbes AA, Logsdon JM Jr. 2017. Retention of core meiotic genes across diverse hymenoptera. J Hered. 108(7):791–806.
Vershinina AO, Kuznetsova VG. 2016. Parthenogenesis in Hexapoda: entognatha and non-holometabolous insects. J Zoolog Syst Evol Res. 54(4):257–1469.
Whiting MF, Bradler S, Maxwell T. 2003. Loss and recovery of wings in stick insects. Nature 421(6920):264–267.
Wu C, et al. 2016. *De novo* transcriptome analysis of the common New Zealand stick insect *Clitarchus hookeri* (Phasmatodea) reveals genes involved in olfaction, digestion and sexual reproduction. PLoS One 11(6):e0157783.
Wu C, Twort VG, Crowhurst RN, Newcomb RD, Buckley TR. 2017. Assembling large genomes: analysis of the stick insect (*Clitarchus hookeri*) genome reveals a high repeat content and sex-biased genes associated with reproduction. BMC Genomics 18(1):884.
Wu TD, Watanabe CK. 2005. GMAP: a genomic mapping and alignment program for mRNA and EST sequences. Bioinformatics 21(9):1859–1875.
Xia XH. 2018. DAMBE7: new and improved tools for data analysis in molecular biology and evolution. Mol Biol Evol. 35(6):1550–1552.
Xu H, et al. 2012. FastUniq: a fast de novo duplicates removal tool for paired short reads. PLoS One 7(12):e52249.
Yang J-S, Lai EC. 2010. Dicer-independent, Ago2-mediated microRNA biogenesis in vertebrates. Cell Cycle 9(22):4455–4460.
Yang Z. 2007. PAML 4: phylogenetic analysis by maximum likelihood. Mol Biol Evol. 24(8):1586–1591.
Yin C, et al. 2016. InsectBase: a resource for insect genomes and transcriptomes. Nucleic Acids Res. 44(D1):D801–807.
Zhang J. 2003. Evolution by gene duplication: an update. Trends Ecol. Evol. 18(6):292–298.

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